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Genotypic characterization of Kaposi's sarcoma-associated herpesvirus in asymptomatic infected subjects from isolated populations

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Molecular epidemiological studies of Kaposi's sarcoma-associated herpesvirus (KSHV) have concentrated on characterization of viral strains in tumour biopsy samples from Kaposi's sarcoma (KS) patients, mostly obtained in the United States and Europe. Tumour biopsies are a convenient source of viral DNA, as they have a high viral load compared to peripheral blood. However, sequences obtained from biopsies may not be representative of viral strains in asymptomatic subjects and information on ethnicity is often not available. Here, a population-based approach has been used to study the molecular and seroepidemiology of KSHV in isolated populations in Ecuador and Botswana. Amerindians in Ecuador had a variable prevalence of KSHV and all strains characterized were of subtype E, based on K1 sequencing. All Amerindian strains had predominant (P)-type K15 alleles and had sequences in both T0.7 and ORF 75 that appeared to be characteristic of these strains. The prevalence of KSHV in two ethnic groups in Botswana was extremely high. K1 sequences from both Bantu and San subjects were mostly of subtypes B and A5, which are typical of African KSHV strains, but the sequence from one San subject did not cluster with any known subtype. Considerable heterogeneity was seen in the T0.7 and ORF 75 genes in the San subjects and one had a minor (M)-type K15 allele. The heterogeneity of the KSHV strains found in these subjects from Botswana contrasts with the homogeneity of KSHV strains in Amerindians, reflecting differences in the evolutionary history of these populations.

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INTRODUCTION

Kaposi's sarcoma-associated herpesvirus (KSHV), also known as human herpesvirus 8 (HHV-8), was discovered in 1994 (Chang *et al.*, 1994) and is the causative agent of Kaposi's sarcoma (KS) (Whitby *et al.*, 1995; Moore *et al.*, 1996), primary effusion lymphoma (PEL) (Cesarman *et al.*, 1995) and multicentric Castleman's disease (Soulier *et al.*, 1995). KS is a rare vascular tumour that was described initially in elderly men of Mediterranean or Jewish descent (Kaposi, 1872). Prior to the AIDS epidemic, incidence of KS was highest in the Mediterranean area (classic KS) (Franceschi & Geddes, 1995) and in east and central Africa (African endemic KS) (Oettle, 1962; Wabinga *et al.*, 1993). Increased incidence of KS was also reported in immunosuppressed organ transplant recipients (iatrogenic KS) (Penn, 1978), providing the first indication of an association with immune suppression. The AIDS epidemic dramatically altered the epidemiology of KS in the United States and northern Europe, where it began to occur frequently in human immunodeficiency virus (HIV)-infected gay men, and in sub-Saharan Africa, where it rapidly became the most common tumour and a major public health problem (Wabinga *et al.*, 1993; Sitas & Newton, 2001).

GenBank accession numbers for the sequences reported in this manuscript are AY329013–AY329033.

KSHV, like other herpesviruses, is believed to have co-evolved with the human species. However, the prevalence of KSHV infection varies geographically, with the level generally reflecting the incidence of KS. Prevalence of KSHV in the general population, as measured by antibody assays, is low (<5 %) in Asia, northern Europe, Australia and the Americas, but higher in Mediterranean countries (4–35 %) and sub-Saharan Africa (20–70 %) (Schulz *et al.*, 2002).

The KSHV genome is highly conserved along most of the unique coding region (approx. 140 kbp), but genes at both ends of the genome have been shown to vary considerably. The amino acid sequence of K1 has been shown to vary by up to 40 %, with the changes concentrated in two hypervariable regions, VR1 and VR2. Five major molecular subtypes of KSHV have been reported, based on K1 sequence analysis: A, B, C, D and E (Cook *et al.*, 1999; Meng *et al.*, 1999; Zong *et al.*, 1999; Biggar *et al.*, 2000). Subtypes A and C occur in Europe, the United States and Australia, whilst subtype C alone predominates across Asia. In Africa, about half of strains sequenced are of subtype B; the remainder mostly belong to subtype A5. Subtype D has been reported in Aboriginal peoples of the Pacific rim, including Taiwan, Australia and Japan (Zong *et al.*, 1999; Meng *et al.*, 2001). We previously reported subtype E in two Brazilian Amerindians (Biggar *et al.*, 2000). The geographical distribution of the subtypes of KSHV led Hayward (1999) to propose that the various subtypes arose over the last 100 000 years, in accord with ancient human migrations and subsequent isolation of discrete populations.

A different kind of evolutionary variability occurs at the right-hand side of the genome. The K15 gene occurs as two distinct alleles with 70 % divergence in their amino acid sequences. The P (predominant) allele is reported more frequently and has been found in association with subtypes A, B, C and D of K1, whereas the M (minor) allele has not yet been described in the rare subtype D (Hayward, 1999). K15 sequencing of subtype E strains has not yet been reported.

Most previous studies of KSHV molecular epidemiology have relied on KS biopsy material. Such material rarely has accompanying sera, sociodemographic information or even reliable information on the ethnicity of the subjects. As these studies examine viral strains that are found in diseased subjects, the findings may not be representative of those in the whole population. Molecular studies on peripheral blood mononuclear cells are considerably more challenging, as viral levels are significantly lower than in tumour biopsies and the virus is difficult to detect at all in asymptomatic infected subjects. However, molecular data from population-based studies are especially important, as correlations with ethnicity, seroprevalence and relative isolation of the population can then be made.

We demonstrated previously that KSHV is hyperendemic among Brazilian Amerindians and identified a unique K1 subtype that we related to the ethnicity, isolation and likely evolutionary history of the subjects (Biggar *et al.*, 2000). In the current study, we extend our observations to Ecuadorian Amerindians and persons of African descent living in Ecuador. For contrast, we also examine two ethnically distinct populations living in Botswana. For all subjects, we examined three gene regions at the right-hand side of the genome: K15, ORF 75 and T0.7, for which data from subtype E strains have not been available previously.

METHODS

Overall strategy. A step-wise screening strategy was used to identify samples that were suitable for genotypic analysis. Sera were initially screened for antibodies to KSHV, to identify KSHV-infected subjects. Matched buffy coat samples were then selected for DNA extraction and real-time PCR analysis, to identify subjects with detectable levels of the KSHV genome. Amplification was then attempted by using nested PCR primers for four gene regions that are likely to provide the most useful evolutionary information (K1, K15, ORF 75 and T0.7/K12).

Subjects and samples. Samples from Ecuador were available from 79 subjects from two different Amerindian tribes, the Huaorani ($n=38$) (Kron *et al.*, 2000) and the Siona ($n=41$) (Benefice *et al.*, 1989). Samples were also tested from 91 Ecuadorians of African descent from the province of Esmeraldas, Ecuador. Blood plasma and buffy coat samples were collected in the course of infectious disease surveys that were organized by the Onchocerciasis Control programme, Ministry of Health of Ecuador, and community health surveys of the Hospital Franklin Tello, Nuevo Rockafuerte, Ecuador. Subjects from Botswana consisted of 160 Bantu and 156 San. Bantu donors were residents of the small town of Ghanzi. Serum and lymphocyte samples were collected in 1987 near a well in the middle of the Central Game Reserve of Botswana, where 1100 nomadic San were known to reside. The donors were all San-speaking and of different tribal affiliations and all indicated that both of their parents were also San people (Ebbesen *et al.*, 1989).

Serological tests. Sera were tested for antibodies to KSHV by using three different tests. An immunofluorescence assay (IFA) measured antibodies to the latent nuclear antigen (LANA) that is encoded by ORF 73, expressed in the KSHV latently infected PEL cell line BCP-1, as described previously (Simpson *et al.*, 1996; Engels *et al.*, 2000b). ELISAs for antibodies to recombinant glycoprotein K8.1 and LANA/ORF 73 were also used. The K8.1 assay has been described previously (Engels *et al.*, 2000a, b). LANA IFA and K8.1 ELISA for the Botswanan samples were performed previously (Engels *et al.*, 2000a). The LANA/ORF 73 ELISA was performed by using full-length baculovirus-expressed protein and a similar protocol. The cut-off for the K8.1 assay was an A_{405} value of $>1{\cdot}5$ and that for the LANA assay was $A_{405}>1{\cdot}0$.

Real-time PCR. DNA was extracted from buffy coat samples by using a Qiagen blood and body fluids kit. KSHV DNA was detected with a quantitative real-time PCR assay by using primers in the KSHV K6 gene, as described previously (de Sanjose *et al.*, 2002). DNA quantity and quality were assessed by using a real-time PCR assay for endogenous retrovirus (ERV)-3 (Yuan *et al.*, 2001). The sensitivity of the K6 assay is three copies per million cells or one copy per 150 ng DNA.

Genotyping PCR. Four regions of the KSHV genome were used to characterize KSHV samples that were positive by PCR. Nested PCR primers used in this study are listed in Table 1. PCR was performed by using Jumpstart Readymix with RedTaq DNA polymerase (Sigma) and the Failsafe PCR system (Epicentre). K1 and ORF 75 gene products were amplified as described previously (Cook *et al.*,

Table 1. KSHV nested PCR primer pairs

Gene locus	Product size (bp)	Primer	Sequence	Reference
K1	868	K1a-f	ATGTTCCTGTATGTTGTCTGC	Cook *et al.* (1999)
		K1a-r	AGTACCAATCCACTGGTTGCG	
	840	K1b-f	GTCTGCAGTCTGGCGGTTTGC	Cook *et al.* (1999)
		K1b-r	CTGGTTGCGTATAGTCTTCCG	
	576	CDC-OF	GACCTTGTTGGACATCCTGTA	Meng *et al.* (2001)
		CDC-OR	GAGTTTCTGGAGTTATATTG	
	363	CDC-IF	TTGTGCCCTGGAGTGATT	Meng *et al.* (2001)
		CDC-IR	CAGCGTAAAATTATAGTA	
K15 (P)	365	K15P-OF	TGCAGGCTTGGTCATGGGTTAC	This study
		K15P-OR	GGGACCACGCTGCAATTAAATG	
	285	K15-3C	ACGCATACATGTACTGCCAC	Kakoola *et al.* (2001)
		K15-4C	CTTTGATATTGCCAGTGGTG	
K15 (M)	486	K15M-OF	TGTTGGTTGCAATGCTTAGGTG	This study
		K15M-OR	GCCTTTGCCAGTTGGAGTTTC	
	370	LGH 2473	CATGCAGCGAGCTTGAGA	Poole *et al.* (1999)
		LGH 2474	CTTTGAGTACTGTTTGTG	
ORF 75	895	KS 1000	CGGTTCGGTGGCATACAGGC	Kakoola *et al.* (2001)
		KS 1034	CTGACTACAGAGGGTGTCCCCG	
	804	LGH 2000	GGAAACAGGGTGCTGTG	Poole *et al.* (1999)
		LGH 2034	CATGGCCTACGACGTCAC	
T0.7	646	LGH 2076	GCTGCAATGTACTGCCATG	Poole *et al.* (1999)
		LGH 2075	CTCCAATCCCAATGCATGGA	
	407	T07-IF	ACTTGGCGTCTGCCGAAGTC	This study
		T07-IR	TAGCGTGCCCTCCCAAAGAG	
	752	T0.7-752-F	TGCAACTGACCATGTTCAGGTG	This study
		T0.7-752-R	CTCCTCCCTCCTCACTCCAATC	

1999; Meng *et al.*, 1999; Poole *et al.*, 1999; Kakoola *et al.*, 2001). The cycling conditions of all nested PCR reactions were similar, differing only with respect to annealing temperatures. PCR cycling conditions consisted of 1 min at 94 °C and 35 cycles of 1 min at 94 °C, 30 s at the appropriate annealing temperature and 2 min at 72 °C. A final 5 min hold at 72 °C ended each assay. Annealing temperatures for the K15 P and M outer primers were 57 and 54 °C, respectively. The T0.7 outer primer conditions were as described previously (Alagiozoglou *et al.*, 2000) and the inner primer annealing temperature was 57 °C. Extreme measures were taken to prevent PCR contamination: separate rooms were used for DNA extractions, reagent preparation and first- and second-round PCR amplification. All equipment and supplies were UV-irradiated. Positive and negative (water) controls were added to each plate.

Cloning and sequencing. PCR products were isolated from agarose gels by using a commercial kit (Qiagen). KSHV gene products were cloned into pGEM-T Easy vector (Promega) or TOPO TA vector (Invitrogen) and sequenced by using M13 primers. Two to five clones were sequenced from each sample to verify the sequence. Samples were sequenced by using an ABI PRISM 310 DNA sequencer (Applied Biosystems). Data were derived from both forward and reverse sequences for all PCR products.

Evolutionary analysis. DNA sequences were aligned by using CLUSTAL X version 1.81 and the CLUSTAL W weight matrix. Neighbour-joining analysis of aligned sequences was carried out in MEGA version 2.1. K1 protein sequences were also aligned in CLUSTAL X version 1.81. The Gonnet 250 weight matrix was used. Phylogenetic analysis was carried out by using neighbour-joining methods in the CLUSTAL X and MEGA version 2.1 software packages. Subtype determinations of the T0.7 region were made by comparison of published nucleotide polymorphisms (Zong *et al.*, 2002) with polymorphisms noted in our sequences.

RESULTS

KSHV seroprevalence in Ecuador and Botswana

Both populations studied in Botswana had a high prevalence of antibodies to both K8.1 and LANA antigens; concordance between assay results was reasonable. In the Bantu, 122/160 subjects (76 %) were positive in the K8.1 assay and 144/160 (90 %) were positive in the LANA IFA. In the San, 137/156 subjects (88 %) were positive in the K8.1 assay and 124/156 (79 %) were positive by LANA IFA.

In Ecuadorian Amerindians, the Huaorani had a high prevalence of antibodies, with 38 of 38 subjects (100 %) reacting in both the LANA IFA and LANA ELISA, whilst 24/38 (63 %) were positive in the K8.1 assay. The Siona had a lower prevalence of antibodies to LANA by IFA (10/41, 24 %) and ELISA (8/41, 20 %) and to K8.1 (10/41, 24 %). Ecuadorians of African descent had the lowest prevalence, with 11/80 (14 %) being positive for K8.1, 14/80 (17·5 %) by LANA ELISA and 10/80 (12·5 %) by LANA IFA.

Real-time PCR

Samples for real-time PCR were selected on the basis of serological testing. By real-time PCR, KSHV was detected in seven of 63 (11 %) Amerindian samples and two of 21 (9·5 %) Ecuadorian subjects of African descent. In samples from Botswana, KSHV was detected in three of 30 (10 %) samples from Bantu subjects and two of 20 (10 %) samples from San people. Raw copy numbers ranged from less than one (qualitatively positive) to six.

K1 analysis

K1 sequence was obtained from three Huaorani and two Siona subjects in Ecuador. From Botswana, four sequences were obtained from Bantu and three from San subjects. Phylogenetic analysis of these sequences, as well as 55 previously published sequences, is shown in Fig. 1. All sequences from Ecuador were of subtype E and clustered with those obtained previously from Brazilian Amerindians. Ecuadorian sequences formed a separate branch within the subtype E grouping and the Huaorani and Siona sequences clustered separately within this branch. Three of the four Botswana Bantu sequences were A5 and the other was of subtype B; this is consistent with previous findings from Africa (Cook *et al.*, 1999; Meng *et al.*, 1999; Lacoste *et al.*, 2000; Kakoola *et al.*, 2001; Zong *et al.*, 2002). Two of the Botswana San sequences were also of subtype B and were similar to the Botswana Bantu sequences. The other San sequence, San 2, did not cluster with any of the known sequences and may represent a novel subtype, although additional sequences will be needed to confirm this. Lacoste *et al.* (2000) have previously described two other highly variant K1 subtypes, K1-Dem and K1-43Ber, but neither of these clusters with the San 2 sequence. San 2 differs from K1-Dem by 17 % and from K1-43Ber by 13 % at the amino acid level.

Other gene regions

K15 sequence was determined for six of seven Ecuadorian Amerindian subjects; all had the P allele. K15 sequence was also determined for two of the four Botswanan Bantu subjects and both had the P allele. One of the San subjects also had a P allele, a second had the M allele and the third could not be determined.

T0.7 sequences from the Ecuadorian subjects were similar to each other, but differed from all others that were available for analysis. We obtained a T0.7 sequence from an African Ecuadorian subject from whom we were unable to amplify the K1 sequence. This sequence was clearly characterized as subtype B, as shown in Fig. 2. T0.7 sequences could not be amplified from any of the four Botswanan Bantu subjects, but were amplified from all three Botswanan San. The two K1 B-subtype San subjects had Q-type T0.7. The Botswanan San subject with the unclassifiable K1 sequence had a conventional B sequence at T0.7.

Fig. 3 shows an analysis of ORF 75 nucleotide variation.

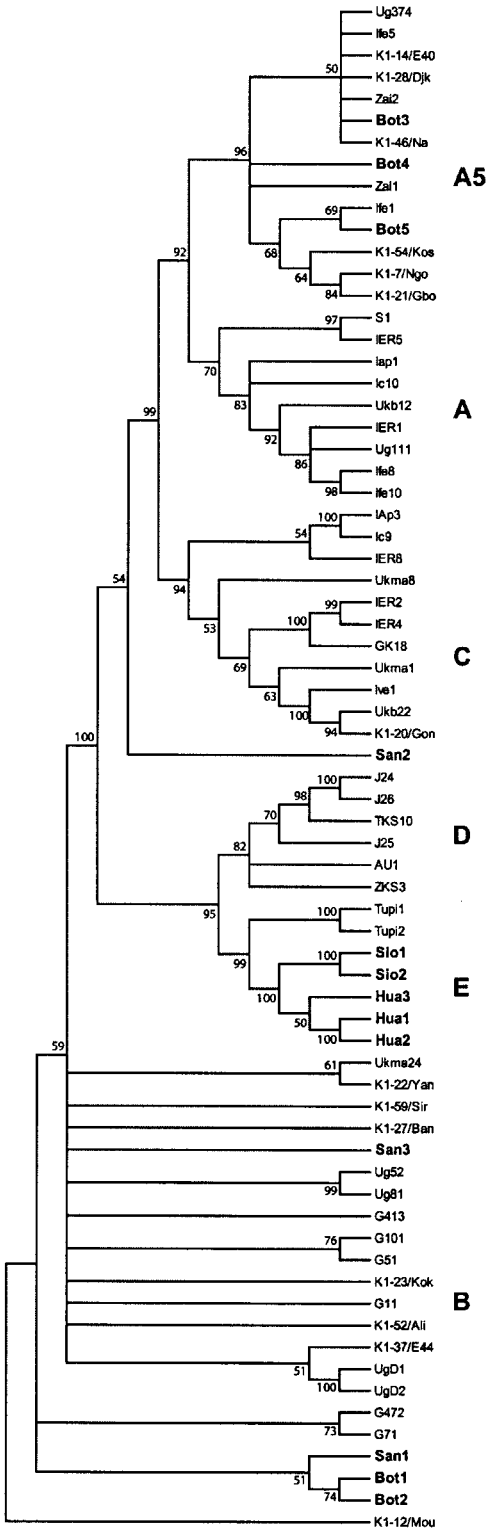

Fig. 1. Cladogram of the translated K1 protein region. Amino acid sequences were aligned in CLUSTAL X version 1.81 and converted to MEGA version 2.1 format. Neighbour-joining analysis using the Poisson correction distance model was carried out in the MEGA software package. One hundred replicate samplings were subjected to bootstrap analysis to create the consensus tree.

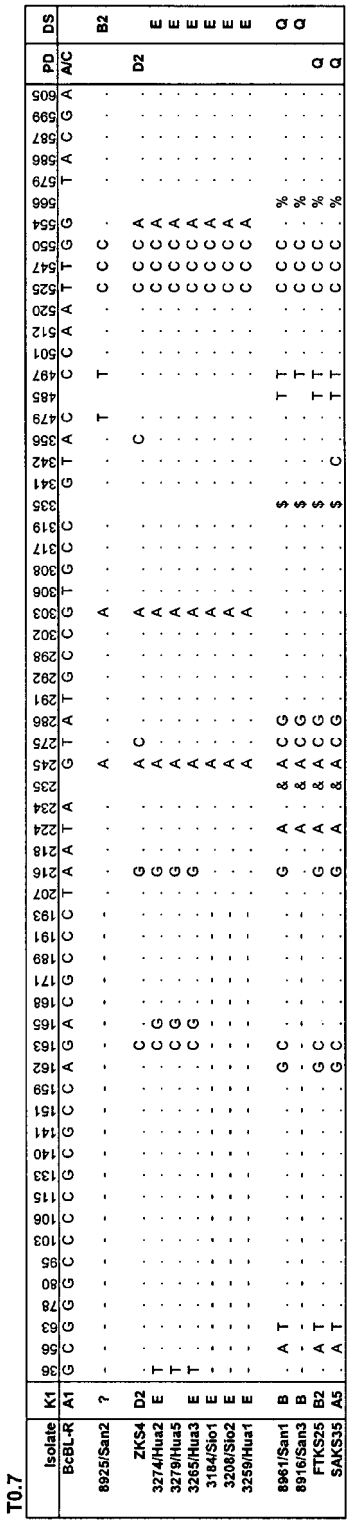

Fig. 2. Nucleotide changes within the KSHV T0.7 region, as compared with published T0.7 sequences. K1 subtypes are indicated. Changes are displayed in the context of variation from BCBL-R as the reference sequence. T0.7 sequences determined previously were published by Zong *et al.* (2002). PD, Previously determined; SD, determined in this study; &, 4 bp deletion; $, 4 bp deletion; %, 5 bp deletion.

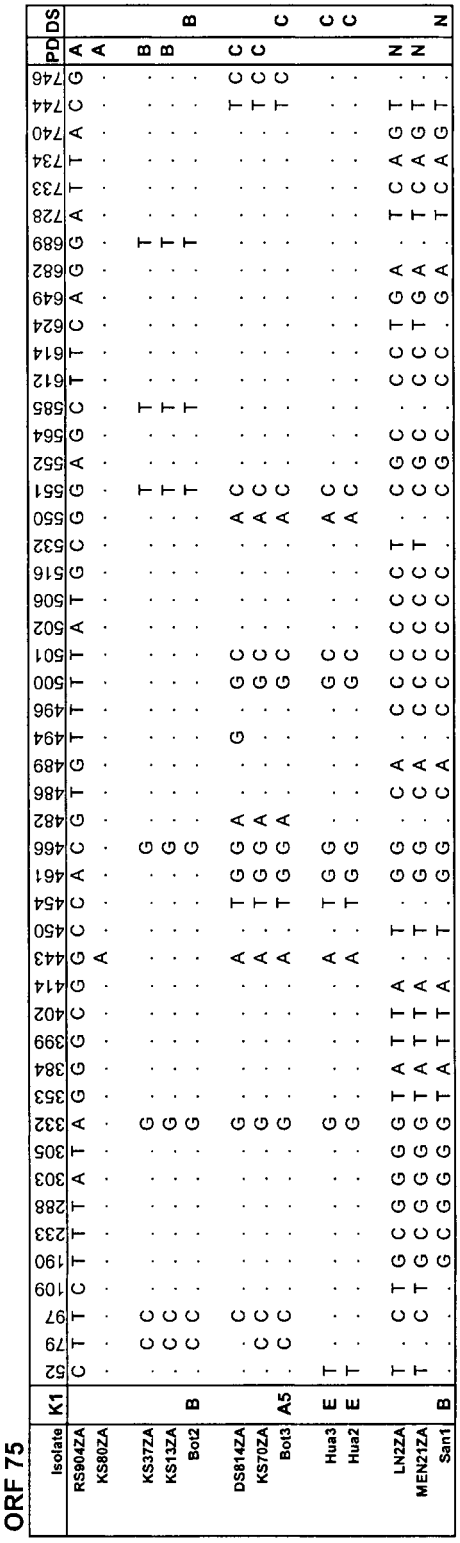

Fig. 3. Nucleotide changes within the ORF 75 locus. New sequences determined in this study were compared with selected sequences available in GenBank. Results are displayed in the context of variation from BCBL-R as the reference sequence. K1 subtypes are indicated. PD, Previously determined; SD, determined in this study.

ORF 75 sequences were amplified from two of seven Amerindian subjects. These sequences clustered together within ORF 75 type C and formed a distinct branch, as shown in Fig. 4. Two of the four Botswanan Bantu subjects yielded ORF 75 sequences, one of which aligned with subtype B and the other with subtype C. ORF 75 was amplified from only one San subject; this sequence was classified as type N.

A summary of the genotyping for all four regions is shown in Fig. 5 and serological and molecular characteristics of the subjects from whom sequence information was obtained are summarized in Table 2.

DISCUSSION

In this study, we report the prevalence and genotypic characterization of KSHV in two ethnically distinct populations in Ecuador and two in Botswana. We reported previously that KSHV was present at hyperendemic levels in Brazilian Amerindians and that prevalence varied considerably, even between villages of the same tribe (Biggar *et al.*, 2000). In the current study, we have found that in one group of Ecuadorian Amerindians, the Huaorani, KSHV infection was apparently ubiquitous in adults, whereas in another, the Siona, KSHV prevalence was somewhat lower. The Huaorani are linguistically unique among Amerindians in Ecuador and are isolated geographically from both the Siona and African Ecuadorian populations. Ecuadorians of African descent had the lowest KSHV prevalence of all groups studied, which is paradoxical given the high prevalence of KSHV in most African populations studied. Indeed, in this study, the prevalence of KSHV was high in both Bantu and San populations studied in Botswana. Low KSHV prevalence in Ecuadorians of African descent is, however, consistent with the reported low prevalence of KSHV in populations of African descent in the Caribbean, South America and the UK and in African-Americans (Goedert *et al.*, 1997; Smith *et al.*, 1999; Lacoste *et al.*, 2000). The reasons for these geographical and ethnic variations in prevalence of KSHV deserve further investigation.

The Ecuadorian Amerindian strains of KSHV were all characterized as subtype E, based on K1 analysis. This subgroup clearly branched separately from subgroup D sequences. As expected, within subtype E, the Ecuadorian strains clustered separately from the Brazilian strains reported previously. The Siona and Huaorani strains also branched independently of each other. All Amerindian strains had the P allele of K15, as do all strains of subtype D that have been reported to date. All T0.7 sequences from K1 subtype E strains had characteristic amino acid changes that were not seen in T0.7 from other subtypes; we designate these as T0.7 type E. The ORF 75 sequences from the strains of subtype E also had characteristic changes and clustered together in a phylogenetic tree. ORF 75 subtype E forms a distinct branch within the C clade of the ORF 75 tree. Additional data may indicate that this is actually a unique subtype.

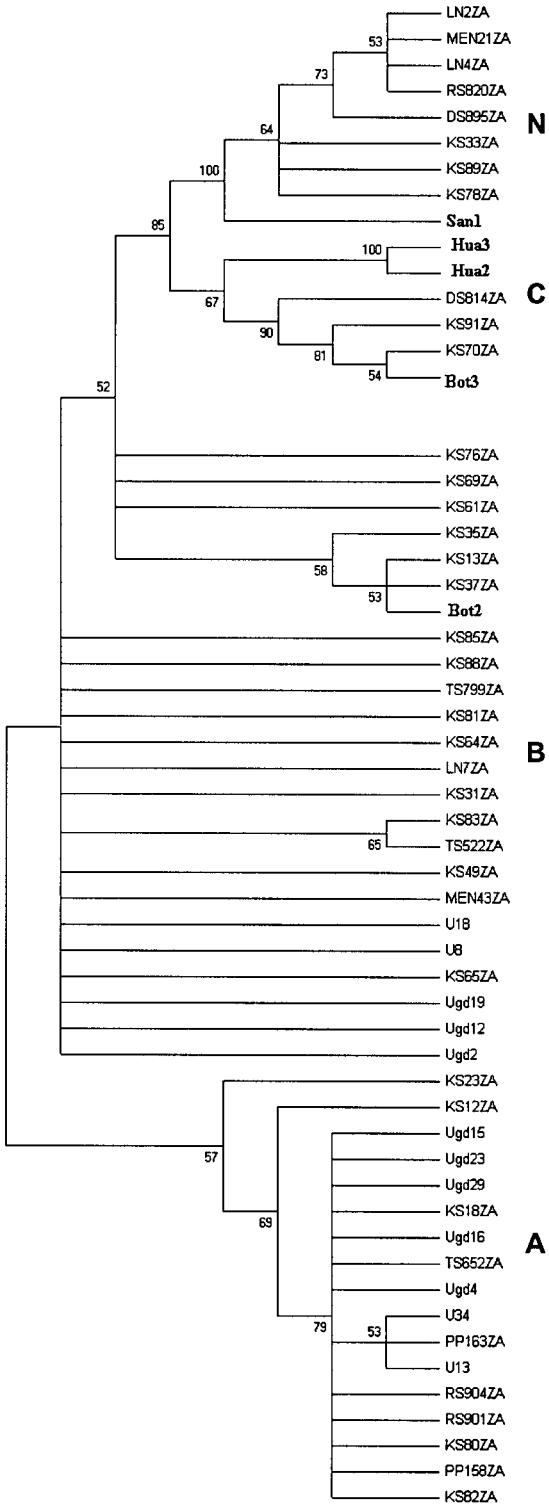

Fig. 4. Phylogenetic analysis of 804 bp of the ORF 75 region and resulting dendrogram, showing relationships for five new isolates. The best tree was determined from 100 replicates in a neighbour-joining algorithm by using the Kimura two-parameter distance model in the MEGA version 2.1 software package. Bootstrap values of $\geqslant 70\,\%$ represent significant branching.

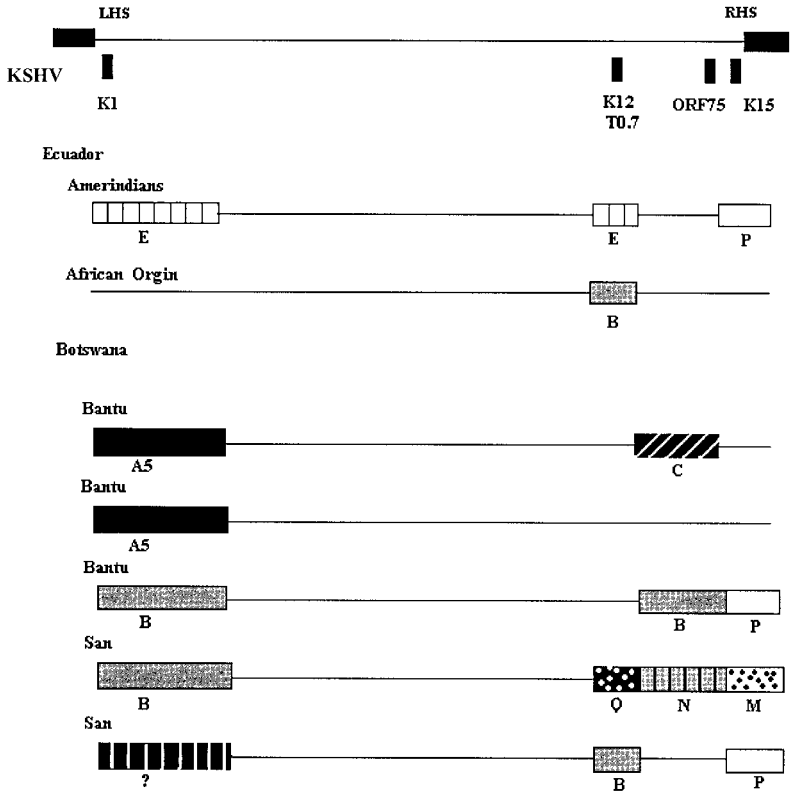

Fig. 5. Summary graphic of the right-hand side (RHS) and left-hand side (LHS) genetic recombination found in this study. Bars indicate the sequence subtype obtained; a straight line indicates a region that was not sequenced or for which no sequence information was obtained.

Recent publications have provided additional data on KSHV subtypes present in South America. Lacoste *et al.* (2000) reported 10 strains from KS patients of African descent in French Guiana; these sequences were typical of those seen in Africa. Meng *et al.* (2001) reported five strains from KS patients of European origin in Argentina; these were characterized as subtypes A or C. Similar data were reported for three AIDS-KS patients in Brazil by Zong *et al.* (2002):

Table 2. Summary of serological and molecular profiles of Ecuadorian and African subjects from whom sequence data were obtained

ND, Not determined; QP, qualitatively positive; UC, unclassifiable.

	Serology					PCR and genotyping			
KSHV strain	**Ethnic origin**	**IFA**	**ORF 73**	**K8.1**	**K6 copy no.**	**K1 (size)**	**T0.7/K12**	**ORF 75**	**K15**
Ecuador									
Hua 1	Huaoroni	+	+	−	1	E (843 bp)	E	ND	P
Hua 2	Huaoroni	+	+	+	1	E (843 bp)	E	E	P
Hua 3	Huaoroni	+	+	+	QP	E (843 bp)	E	E	P
Hua 4	Huaoroni	+	+	+	QP	ND	ND	ND	P
Hua 5	Huaoroni	+	+	−	1	ND	E	ND	ND
Sio 1	Siona	+	+	+	1	E (843 bp)	E	ND	P
Sio 2	Siona	+	+	+	1	E (843 bp)	E	ND	P
Botswana									
Bot 1	Bantu	−	ND	+	1	B (363 bp)	ND	ND	P
Bot 2	Bantu	+	ND	+	QP	B (363 bp)	ND	B	P
Bot 3	Bantu	+	ND	+	6	A5 (843 bp)	ND	C	ND
Bot 4	Bantu	−	ND	+	1	A5 (843 bp)	ND	ND	ND
San 1	San	+	ND	+	QP	B	Q	N	M
San 2	San	+	ND	+	1	UC	B2	ND	P
San 3	San	+	ND	+	QP	B	Q	ND	ND

two were A and one was C. Together with data reported in this study, these reports indicate that in South America, there has been little KSHV spread between ethnic groups since colonization. It is unclear at present whether this is a result of limited contact between ethnic groups or preferential transmission of KSHV to genetically related hosts, as we hypothesized previously (Biggar *et al.*, 2000).

Subtype E sequences were related most closely to subtype D, which has been reported from Asia, the Pacific islands and Australia. The first subtype D sequences were identified in classic KS patients who belonged to the Hwalian ethnic group in Taiwan (Zong *et al.*, 1999). Additional sequences were reported from the Pacific islands, Australia and the Japanese island of Hokkaido (Meng *et al.*, 2000, 2001; Zong *et al.*, 2002). Ethnicity was not reported for most of these subjects. In our view, it is likely that before the expansion of the Chinese people, the populations of Asia and the Pacific islands were infected with subtype D and E viruses that are now maintained only in Aboriginal populations. Further studies are needed to determine whether subtype D strains will also be found in Amerindians or, conversely, whether subtype E strains are still present in isolated populations of Asia.

Strains characterized from Botswana mostly belonged to typical African subtypes. Two of the four strains from Bantu subjects were of subtype B and the remaining sequences were of subtype A5. Two of the San subjects also had subtype B strains and these clustered closely with the sequences from Bantu subjects. This would suggest that KSHV transmission has occurred between these ethnic groups, unlike the findings in South America. One San subject had a unique K1 sequence that did not cluster with any of the major subtypes. The San peoples studied in this survey represented very rural subjects (typically called bushmen), this group being among the most isolated of the Khoisan ('click') language speakers that remain in Africa. This virus, therefore, may represent the type present in the San prior to the Bantu and European expansion into southern Africa over the past 2000 years, a migration that marginalized the San and other Khoisan-speaking ethnic groups to the southern part of Africa. Further studies of other minority ethnic groups in Africa are needed to substantiate this speculation.

Previous reports have noted considerable heterogeneity in sequences at the right-hand side of the KSHV genome in subjects from Africa (Alagiozoglou *et al.*, 2000; Lacoste *et al.*, 2000; Kakoola *et al.*, 2001; Zong *et al.*, 2002). Our data are consistent with these observations. Two San subjects had type Q T0.7 sequence polymorphisms and one also had an ORF 75 type N sequence. Type N and Q sequences have been reported previously in KSHV strains from South Africa. Our data may suggest that the source of these divergent genes originated in KSHV strains of the Khoisan peoples. Consistent with previous reports (Meng *et al.*, 1999; Zong *et al.*, 1999), we observed no episodes of dual infection by different subtypes of KSHV, suggesting that it is probably a rare event.

One San subject had an M allele of K15, which has been reported to be rare in Africa by some groups (Kakoola *et al.*, 2001; Zong *et al.*, 2002) and common by others (Lacoste *et al.*, 2000). A variant K15 M strain has been reported in a Ugandan KS patient, which was designated M′ (Kakoola *et al.*, 2001). The K15 M sequence present in one of our Botswanan San subjects aligns more closely with the M′ sequence than with other previously reported M sequences.

In summary, we have studied both the prevalence and molecular characteristics of KSHV in isolated populations in Ecuador and Botswana. In Ecuador, the prevalence of KSHV varied between the ethnic groups studied, being lowest in Ecuadorians of African descent. The Amerindian KSHV strains characterized were of subtype E at all loci and all had the K15 P allele. In Botswana, we observed a high prevalence of KSHV in both ethnic groups studied. The K1 sequences detected in the Bantu and San subjects were mostly of subtypes A5 and B, which are typically found in Africa; however, we identified a San subject with a K1 gene that does not cluster with any of the major KSHV subtypes. We observed considerable heterogeneity in the more conserved genes at the right-hand side of the genome in the San subjects, suggesting that these divergent genes may have arisen in Khoisan KSHV strains.

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